INFLUENCE OF ENVIRONMENTAL FACTORS ON TENUAZONIC ACID PRODUCTION BY *Epicoccum sorghinum*: AN INTEGRATIVE APPROACH OF FIELD AND LABORATORY CONDITIONS

Rodrigo C. Oliveira [a], Hang N. Nguyen [b], Carlos A. Mallmann [c], Rogerio S. Freitas [d], Benedito Correa [a], Debora F. Rodrigues [b]*

[a] Laboratory of Mycotoxins and Toxigenic Fungi, Department of Microbiology, University of Sao Paulo, 05508-900, SP, Brazil.

[b] Department of Civil and Environmental Engineering, University of Houston, Houston, 77204-400, TX, USA.

[c] Laboratory of Micotoxicological Analysis, Federal University of Santa Maria, Santa Maria, 97105-900, RS, Brazil.

[d] São Paulo Agency for Agribusiness Technology, Votuporanga, 15500-970, SP, Brazil.

* corresponding author: Debora F. Rodrigues, Ph.D.
Cullen college of Engineering Bldg 1 room N107
4726 Calhoun Rd, Houston, TX 77204
E-mail: dfrigirodrigues@uh.edu
Phone: +1-713-743-1495
Fax: +1-713-743-4260

Abstract

Sorghum is the fifth most cultivated and consumed grain in the world. However, this grain is frequently contaminated with toxins from fungi. The present study evaluated the effects of environmental factors on tenuazonic acid (TeA) production by *Epicoccum sorghinum* in the field and in controlled laboratory conditions. In this study, 50 sorghum grain samples were collected from summer and autumn growing seasons and analyzed for TeA contamination using LC-MS/MS. To further understand the ecophysiology of this fungus, an isolated strain of *E. sorghinum* from the field was investigated for its development and TeA production under controlled environmental conditions in the laboratory. In the ecophysiological investigation, the effects of water activity (0.90, 0.95, 0.99) and temperature (18, 22, 26 and 30 °C) were evaluated on the radial growth, enzymatic production and expression of *TAS1*, which is the gene involved in TeA production. Results showed that in the field, the summer season presented the highest TeA average level in the grains (587.8 µg/kg) compared to level found in the autumn (440.5 µg/kg). The ecophysiological investigation confirmed that *E. sorghinum* produces more actively TeA under environmental conditions simulating the summer season. Optimum growth, maximum *TAS1* gene expression, and higher extracellular enzymatic production were observed at 26°C with a water activity of 0.99. Pearson correlation analyses showed that the production of TeA highly correlates with fungal growth. The present study demonstrates that abiotic factors in a combined approach of field and laboratory conditions will assist in predicting the driving environmental factors that could affect growth of *E. sorghinum* and TeA production in sorghum grains.

Keywords: Sorghum; Ecophysiology; Gene expression; *Phoma sorghina*; LC-MS/MS

1. Introduction

Sorghum is a crop with increasing global importance in food, animal feed, energy production and other diverse industrial uses [1]. According to the USDA [2], sorghum production worldwide in 2016/2017 was estimated to be 62.36 million metric tons, which represented an increase of 4.03 million tons compared to 2015/2016. However, one of the major biotic constraint to sorghum improvement and productivity is fungal contamination, especially in the field when grain development can coincide with favorable weather conditions for fungal growth and subsequent mycotoxin formation [3]. Among the diverse fungi reported to contaminate sorghum grain, *Epicoccum sorghinum* (also known as *Phoma sorghina*), is commonly described as one of the major fungal contaminants at pre- and post-harvest stages [3,4,5,6]. This fungus is a producer of tenuazonic acid (TeA), a mycotoxin that is considered a potent inhibitor of protein biosynthesis and has been reported to cause acute toxicity to several animals [7,8,9]. Moreover, TeA is considered to be responsible for causing Onyalai, a human hematological disorder [10].

In recent years, TeA has gained considerable attention in the scientific community due to their frequent contamination in food and animal feed. However, most available data about TeA occurrence has been linked to the presence of *Alternaria* spp., while little is known about TeA production by E. *sorghinum*. In 2011, the European Food Safety Authority [11] published a report on the risks of *Alternaria* toxins for animal and public health. TeA was considered unlikely to be of human health concern. However, only a few data on the occurrence of TeA in food commodities were available at that time and the TeA uptake estimation was based on chronic dietary exposure of adult populations [12]. More recently Rychlik et al. [13] determined that TeA contamination on

sorghum/millet-based food represents a potential risk to infant health. These recent findings raised more concerns about health and safety of the TeA mycotoxin.

As part of effective crop protection strategy, the understanding of the ecophysiology of fungal pathogens as well as the occurrence of mycotoxins triggered by environmental conditions, are essential to better understand and prevent mycotoxin contaminations. Our recent study described the identification of the TeA biosynthetic gene (*TAS1*) in *Epicoccum sorghinum* [14]. This study enabled us to investigate the TeA biosynthesis in more detail in the present study. Moreover, there are no data on the ecophysiology of *E. sorghinum* as well as the effect of environmental factors linking the TeA occurrence in the field.

Therefore, this study aims to integrate molecular and ecological approaches to better understand the key abiotic factors in TeA production by *E. sorghinum*. For that, we evaluated whether growing seasons (summer and autumn) affect the occurrence of TeA mycotoxin in sorghum grains. In Brazil, these seasons have different temperatures and humidity levels. Therefore, controlled laboratory experiments simulating field conditions with a field isolate of *E. sorghinum* was also investigated under different temperatures and water activities (a_w). These two environmental parameters were correlated with fungal growth and gene expression of TeA as well as enzymatic activity.

2. Material and methods

2.1. Field study

Cultivation and sampling of sorghum grains occurred from November 2012 to May 2013 to determine the influence of growing seasons (summer and autumn) on the occurrence of TeA in sorghum grains. A total of 50 sorghum grain samples (25 from each growing season) were collected in a sorghum plantation owned by the São Paulo Agency for Agribusiness Technology (APTA) (Votuporanga, the state of São Paulo, latitude 20º 28’

S, longitude 50º 04' W). APTA uses the best practice for sorghum cultivation in Brazil. The cultivar DKB 550 (Dekalb seeds), commonly grown in Brazil, was used for both growing seasons. The sowing of summer and autumn crops occurred in November of 2012 and February of 2013, respectively, with a distance of 0.5 m between rows and a final population of 180 000 plants ha^{-1}.

For sampling, a stratified random sampling design was followed [15]. The experimental area used for both growing seasons was divided into five uniform plots. The samples were composed of five panicles randomly collected from each plot. The panicles were hand-harvested and pooled, and 100g of each sample was immediately analyzed for fungal contamination and water activity (a_w) and then stored at 4°C until mycotoxin analyses. The water activity was determined by automatic analysis using an Aqualab CX-2 apparatus (Decagon Devices, Pullman, WA, USA).

The prevailing climatic factors during the sample collection periods, including temperature (°C) and precipitation (mm), were monitored by the Climatological Station (CIIAGRO), located at Votuporanga, SP, Brazil. The data were obtained daily during both growing seasons, from sowing to harvest time.

2.2 Frequency of *E. sorghinum*

From each sample, 30 sorghum grains were surface-disinfected with a sodium hypochlorite solution (1%) and then plated (10 grains per Petri dishes) onto a potato dextrose agar (Oxoid, Basingstoke, UK) supplemented with chloramphenicol (100 mg l^{-1}). The plates were incubated at 25°C in the dark for five days. Colonies of *Epicoccum* spp. developing from the sorghum grains were then counted and morphologically identified according to *Phoma* Identification Manual [16]. Colonies were identified using morphological criteria, such as colony aspect and micromorphological features. The identification at the species level was performed using molecular analysis of ITS region

of the rRNA gene, as described by Oliveira et al. [17]. The results are reported in percentage of infected grains.

2.3. Tenuazonic acid analysis

The sorghum samples were analyzed for TeA mycotoxin contamination utilizing high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) based on the method described by Oliveira et al. 2017 [17].

Briefly, samples of 5 g of ground sorghum grain were homogenized for 90 min with 15 mL of acetonitrile:water (1:1, v/v), acidified with 110 µL of formic acid. After centrifugation (5 min at 4000 rpm), an aliquot of 100 µL of supernatant was taken and diluted with 900 µl of water. TeA was determined by API 5000 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an Ion Electrospray Ionization (ESI) source in the negative ionization mode. The column used was a 50 mm x 4.6 mm x 1.8 µm SB C-18 (Agilent). Multiple-reaction monitoring (MRM) was used for TeA determination. The precursor peak of TeA (*m/z* 196.0) and two products peaks *m/z* 139 (DP - 75, CE -26 V, CXP -19 V) *m/z* 112.0 (DP – 75, CE -34 V, CXP -15 V) were monitored to accomplish both quantitation and qualification criteria. A binary gradient at a flow rate of 0.4 mL/min was performed with solvent A (water) and solvent B (methanol), following the conditions described by Oliveira et al. 2017 [17].

2.3.1 Method validation

Method performance characteristics such as recovery, linearity, limits of detection (LOD) and limits of quantification (LOQ) were evaluated. For the recovery test, free TeA samples of sorghum grain were spiked with a standard solution at two concentrations (100 µg/kg and 200 µg/kg), in triplicates. A calibration curve was obtained using six TeA standard concentrations (20, 200, 400, 600, 800, and 1000 µg/kg).

The mean recoveries for both concentrations were 96.3% and 99.7%, respectively. Good linearity was achieved with a correlation coefficient higher than 0.999. LOD (signal to noise ratio = 3) and LOQ (signal to noise ratio = 10) were 4 and 10 µg/kg, respectively and showed the suitability of the method for the TeA determination in sorghum grains.

2.4. Assessment of eco-physiology of *E. sorghinum* isolated

2.4.1. Fungal strain and growth media

The TeA producer strain *Epicoccum sorghinum* P48 was used in this study. It was previously isolated from the same sorghum field studied and the species identification and ability to produce TeA was confirmed using molecular tools and LC-MS/MS [17]. The strain was grown on oatmeal agar (Difco, Detroit, Michigan) for 7 days at 25 °C and maintained in 15% glycerol at −80 °C in the Department of Microbiology at the University of Sao Paulo. The nucleotide sequence is available in the GenBank database under the accession number: KT31009.

For the experimental conditions, such as growth rate, *TAS1* gene expression and enzymatic production a sorghum-base medium was used. This medium was prepared by blending 4% (w/v) ground sorghum grains in sterile water and then adding 2% (w/v) agar [18]. Except for enzymatic analysis, in which a sorghum-base broth was used instead of agar, since the API-ZYM method cannot be used in agar plates. For the a_w, we used the sorghum-base agar supplemented or not with glycerol to obtain a_w of 0.90 (280 ml/L); 0.95 (152 ml/L) and 0.99 (nothing added). Water activity was determined by an Aqualab CX-2 apparatus (Decagon Devices, Pullman, USA).

2.4.2. Growth rate

A 5-mm-diameter mycelial disk from the margin of a 7-day-old growing colony of *E. sorghinum* strain was used to centrally inoculate all treatments. The plates containing

191 medium with different a_w values (0.90, 0.95, 0.99) were incubated at 18, 22, 26 and 30°C
192 for 7 days; the experiments were performed in triplicate. Assessments of radial growth
193 were made daily by measuring two right-angled diameters of the colonies. The diameters
194 were plotted against time, and radial growth rates (mm/day) were obtained from the slope
195 of linear regressions [19].
196
197 **2.4.3. *TAS1* gene expression**
198 **2.4.3.1. RNA extraction and cDNA synthesis**
199 Petri plates (diameter 90 mm) containing autoclaved media treatments were overlaid with
200 sterile cellophane membranes to facilitate removal of the mycelium for RNA extractions.
201 The total RNA was extracted from mycelium of each of the three replicates using the
202 RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's
203 instructions. First strand cDNA was synthesized using the High Capacity cDNA Reverse
204 Transcription kit (Applied Biosystems, Carlsbad, CA, USA), according to the
205 manufacturer. The synthesis was performed in a Veriti thermal cycler (Applied
206 Biosystems) using the following conditions: hybridization step of 10 min at 25 °C, reverse
207 transcriptase (RT) step of 120 min at 37 °C and 5 min at 85 °C. The cDNA samples were
208 stored at – 20 °C.
209
210 **2.4.3.2. Quantitative real-time PCR (qRT-PCR)**
211 The transcription profiles of the TeA biosynthetic gene *(TAS1*) and of β-tubulin gene
212 (*TUB2*), as a reference gene, were analyzed by using qRT-PCR. The primers were
213 designed using the NCBI tool for qRT-PCR Primer Design
214 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in table 1. For the *TAS1*
215 primer design, a search in the NCBI database was performed (www.ncbi.nim.nih.gov) for
216 similar proteins of the *TAS1* gene recently obtained from the sequenced genome of *E.*

sorghinum P48 strain [14]. Homolog proteins of *TAS1* were found sharing over 83% identity with domain structures of *TAS1* gene (C–A–PCP–KS). Base on that, *TAS1* specific pairs of primer for *E. sorghinum* were designed from a sequence of KS domain found in *E. sorghinum* genome, which was described as an indispensable domain for *TAS1* activity. The pair of primers used to amplify *TUB2* gene was designed from a *TUB2* sequence of the *E. sorghinum* genome in a similar manner as the *TAS1* primers.

The qRT-PCR assays were carried out using 2X Power SYBR Green PCR master-mix (Invitrogen, U.S.A.), according to the manufacturer. Primer optimization was performed following the manufacturer guidelines. The comparative ΔΔCt method was used for the analysis of the qRT-PCR including the melting curve (Step One Plus Real time PCR system, Applied Biosystems). The PCR efficiencies for both genes were performed using the suggested protocol from the manufacturer. Briefly, the cDNA of positive control templates was diluted to the factor of 10 with 6 points and *TAS1* and TUB2 primers were run at the same time to check for the qPCR validation and efficiency. The qPCR parameters obtained in this study were acceptable to the conditions required to quantify the relative gene expression using the $2^{-\Delta\Delta CT}$ method (Fig. S1) [20]. The ΔCt and ΔΔCt were calculated from the Ct value obtained from the results of qRT-PCR following the calculation described by the manufacturer of Applied Biosystems. Samples were performed in triplicate at the same time with negative controls (without cDNA template) and using the thermal cycle and the reaction mix described in Table 1. The results were expressed in terms of fold change vs samples.

2.4.4. Extracellular enzymatic production

The effects of different temperatures on the extracellular enzymatic production of *E. sorghinum* were evaluated by API-ZYM® system (Bio-Me´rieux, Marcy l'Etoile, France). Five culture discs (5 mm) from each of *E. sorghinum* isolates were transferred

to flasks containing sorghum-base broth and were incubated at 22°C for 10 days [21,22]. The growth media were filtered with 0.22 µm Millex filters (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Ireland) and the filtrates were used to determine production of 19 extracellular enzymes with the API-ZYM® system (Bio-Merieux, Marcy l'Etoile, France). Briefly, 65 µL of each fungal culture broth were deposited in the 20 well-plates, and the plates were maintained at 30°C for 4 h in the dark. Thereafter, a drop of ZYM A reagent (25 g of Tris–hydroxymethyl aminomethane + 11 mL of hydrochloric acid 37% + 10 g of sodium lauryl sulphate + 100 mL of H_2O) and ZYM B reagent (0.12 g 'Fast Blue BB' + 40 mL of methanol + 60 mL dimethyl sulphoxide) were added to each well. The results were determined in nanomoles (nmol) of the hydrolyzed substrate according to the intensity of the color reaction on a scale of 1–5, i.e., 1 = 5 nmol, 2 = 10 nmol, 3 = 20 nmol, 4 = 30 nmol and 5 = >40 nmol. All of the measurements were taken in duplicate, and the sorghum-base broth was used as the negative control.

2.5. Statistical analysis

To compare the frequency of *E. sorghinum* and TeA content between summer and autumn growing seasons, t-test was used ($P < 0.05$). The effects of temperature, a_w and their interaction were evaluated by the two-way ANOVA for growth rate and *TAS1* gene expression. The differences among mean values were performed by Tukey`s Multiple Comparisons Test at a 99% confidence level. To better correlate the temperature and a_w with growth and TeA production we used Pearson correlations. All the analysis was performed in OriginTM software (OriginLab, Inc.).

3. Results

3.1. *E. sorghinum* and TeA occurrence in the field

Mycological and mycotoxin analysis of the sorghum grains grown during the two-growing season (summer and autumn) are shown in Table 2. In both growing seasons *E. sorghinum* was the major fungal species isolated, however, the natural variation of environmental factors of each season affected the frequency of *E. sorghinum*. For instance, 74% and 87.4% of the sorghum grains samples from summer and autumn season, respectively, were contaminated with *E. sorghinum* (P = 0.00005224). The highest average level of TeA was found in sorghum grains cultivated during the summer (587.8 µg/kg), with contamination levels ranging from 165.4 to 1647.5 µg/kg; while in autumn, the average of TeA levels was 440.5 µg/kg (198.6 - 1154.9 µg/kg) (P = 0.5680). These growing seasons present very distinct environmental conditions (Table 2). For instance, the summer temperature average (26.3°C) was 4 °C higher than in autumn (22.4 °C). The total rainfall was similar in both seasons (10.5 mm - summer; 9.2 mm - autumn). However, the percentage of rainy days observed during the summer (38.3%) was higher than in autumn (16.6%). Precipitation is known to have a direct effect on the a_w levels in sorghum grain. This was confirmed by the samples collected during the two seasons. For instance, in the summer the a_w was around 0.98, as opposed to autumn that was 0.55.

3.2 Ecophysiology of *E. sorghinum*

To better understand the impacts of a_w and temperature in the development and production of TeA by this fungus, laboratory controlled experiments were performed with different conditions of a_w and temperatures. These conditions were selected based on the climatological data collected in the field during summer and autumn seasons.

3.2.1. Mycelium growth

Fig. 1 shows the radial growth rate of the *E. sorghinum* strain on sorghum media in response to different a_w (0.90-0.99) and temperatures (18-30°C). The results indicated

293 that mycelium growth occurred over the temperature and a_w ranges investigated. The
294 optimum growth condition occurred at 0.99 a_w and 26°C (5.7 mm/day). The results also
295 confirmed that the combination of the lowest temperature and driest conditions (0.90 a_w;
296 18°C) tested were less favorable to *E. sorghinum* growth compared to higher values (0.99
297 a_w; 30°C). Statistical analysis of variance (ANOVA) showed that all single factors (a_w,
298 temperature, and their interaction) were significant on the radial growth rate ($P < 0.01$),
299 except for 22°C and 30 °C at 0.95 a_w. The correlations of temperature and a_w with growth
300 and TeA production was also confirmed by the Pearson correlation (Fig. 2). In agreement
301 with the high TeA level found in sorghum grain grown on summer, a high and positive
302 correlation was found between growth and *TAS1* gene expression in a combination of hot
303 and humid conditions (26 - 30 °C and 0.99 a_w) (Fig. 2; Table S1).
304
305 **3.2.2. *TAS1* gene expression**
306 The results of the relative gene expression of the *TAS1* in *E. sorghinum* strain grown on
307 sorghum medium under different combination of temperature and a_w is shown in Fig. 3.
308 The highest expression of *TAS1* was observed at 26 °C and at 0.99 a_w, which was also the
309 same optimum condition found for mycelium growth and agree with the highest TeA
310 levels found in sorghum grains grown on summer season. These parameters also
311 correlated well in the Pearson correlation with 0.87 and 1.00 for the 0.99 a_w and 26 °C,
312 respectively (Fig. 2; Table S.1.). At lower temperatures (18°and 22°C), the transcriptional
313 level of the *TAS1* was noticeably lower compared to the 26°C, except for 0.90 a_w. At
314 30°C, a considerable decrease of transcription was observed at 0.90 a_w. However, at 0.99
315 a_w, the expression followed the same pattern of 18° and 22°C. Moreover, it is interesting
316 to note that the influence of a_w in *TAS1* gene expression was more pronounced at higher
317 than at lower temperatures. Statistical analysis of variance (ANOVA) showed that all

single factors (a_w, temperature, and their interaction) were significant ($p < 0.01$) on the *TAS1* gene expression, except for 0.90 a_w at 18, 22 and 26 °C.

3.2.3. Enzymatic production

The effect of temperature in the extracellular enzymatic production of *E. sorghinum* is shown in Fig. 4. The following five extracellular enzymes were produced: alkaline phosphatase, acid phosphatase; naphthol-AS-BI phosphohydrolase; α-glucosidase and N-acetyl-β-glucosaminidase. These five enzymes were only simultaneously produced at 22°C and 26°C. A thermal stress was observed at the lowest (18ºC) and highest (30ºC) temperatures tested. Two out of the five enzymes were inhibited, alkaline phosphatase and N-acetyl-b-glucosaminidase at 18°C and naphthol-AS-BI phosphohydrolase and α-glucosidase at 30°C. Moreover, production of acid phosphatase and naphthol-AS-BI phosphohydrolase increased at 18° C, when compared to the other temperatures. We also observed a pigment production when the colonies were cultivated in lower temperatures, with the highest production at 18 °C.

These results suggested that intermediate temperatures (22 and 26 °C) could lead to a more effective grain colonization by this species, and may explain the high frequency of *E. sorghinum* found in sorghum grains grown under the two-growing seasons (22 °C autumn and 26 °C summer).

4. Discussion

It is well known that environmental factors modulate fungal development and mycotoxin production, especially those related to climate change, which directly cause an impact on food security and quality [23]. In Brazil, sorghum cultivation occurs mainly as succession planting, especially in succession to summer crops, from September to March. In a recent study, based on the zoning of climatic conditions, it was determined that 53% of the

344 national territory are able to produce this crop, with the potential to increase the
345 production up to 54 times [24]. However, there is no data on the influence of sowing
346 period concerning food safety aspects, such as fungal and mycotoxin contamination.
347 In our study, natural TeA contamination was found in sorghum grain grown in both
348 growing seasons (summer and autumn). However, environmental conditions prevailing
349 in the summer showed to be more favorable to TeA contamination. During this season,
350 the sorghum grains were exposed to warm and rainy weather with a mean temperature 4
351 °C higher than autumn and precipitation percentage more than double the value of the
352 autumn season (Table 2). The precipitation clearly influenced the a_w in the sorghum
353 grains, keeping high a_w levels (0.91) during the summer season. According to Srivastava
354 et al. [25] changing planting or harvest dates of sorghum crops can be an effective and
355 low-cost option to reduce pests and diseases in crops,
356 Currently, there are no regulations worldwide for TeA in food; however, the average of
357 TeA level found in sorghum grains grown in summer (587.8 µg/kg) was higher than the
358 regulatory limit proposed by Bavarian Health and Food Safety Authority (500 µg/kg the
359 TeA content in sorghum-based infant food) [13]. Therefore, a correct choice of sowing
360 date, especially during a cooler and dry season, can play an important role in reducing the
361 TeA contamination in sorghum crop.
362 To our knowledge, very little information on the ecophysiology of *E. sorghinum* is
363 available. The only previous work that evaluated the effects of temperature and pH on
364 growth of *P. sorghina* strains demonstrated that the optimum temperature for mycelium
365 growth was 28°C [26]. On the other hand, *Alternaria* spp., an important TeA producer,
366 has been studied extensively over the last decade. Several studies have examined the
367 response of *Alternaria* spp. under different environmental conditions [19,27] and
368 interestingly, comparing to our results, *E. sorghinum* have a similar pattern of growth and

TeA production, especially at elevated a_w and temperature conditions (≥0.95; 26°C). For instance, the optimum condition for mycelia growth of *Alternaria* has been observed in a range of 25~30°C and 0.98~1 a_w [27]. In terms of TeA production, Megan and Baxter [28] demonstrated that 0.99 was the optimum a_w level for TeA production by *A. alternata* on sorghum grain. Similar results were found by Oviedo et al. [29], who reported maximum TeA production at 0.98 a_w and between 25 and 30° C for strains of *A. alternata* on soybean-based agar.

Epicoccum sorghinum is an important mold fungus infecting sorghum grains. To overcome the barrier of the grain tissue, an array of extracellular hydrolytic enzymes are produced to facilitate the penetration and subsequent infection on the host tissue [30]. However, thermal stress experienced by the microbial pathogen may impact the dynamics of host/pathogen interactions and ultimately resulting in changes in virulence [31]. Results showed that temperature plays an important role on the enzymatic expression of *E. sorghinum* (Fig. 4). Alkaline phosphatase and naphthol-AS-BI-phosphohydrolase, important enzymes for the degradation of organic phosphorus compounds found in the lipid layer of the plant cell plasma membrane, were inhibited at 18°C and 30°C respectively, as well as N-acetyl-β-glucosaminidase at 18°C [32]. Moreover, α-glucosidase, which hydrolyzes disaccharides and is usually involved in the plant polysaccharides degradation, was inhibited at 30°C [33]. The results suggested that intermediate temperatures (22 and 26 °C) led to maximum enzymatic production, which could lead to more effective grain colonization by this species.

In the case of the TeA biosynthetic gene, this is the first study evaluating the effects of environmental factors on *TAS1* gene expression. *TAS1* was recently described in *Magnaporthe oryzae*, a pathogen of rice, and consists of an NRPS (non-ribosomal peptide

393 synthetase) with an unique type PKS-KS domain (polyketide synthase) that
394 biosynthesizes TeA from isoleucine and acetoacetyl-CoA [34].
395 In the recently sequenced *E. sorghinum* genome, *TAS1* was determined to be highly
396 conserved and had an identical domain described in *M. oryzae*, which is in agreement
397 with the capability of this species to produce TeA [14]. In the current work, temperature
398 and a_w showed a significant effect on *TAS1* expression. It is noticeable that the best
399 condition of the *TAS1* gene expression (26 °C and 0.99 a_w) matches the higher level of
400 TeA detected in the grains grown under the warm and humid conditions found in the
401 summer season. Moreover, the highest *TAS1* expression range includes the optimal
402 conditions for growth and extracellular enzymatic production, suggesting that TeA
403 production by *E. sorghinum* also occur within the range of environmental conditions most
404 favorable for sorghum grain colonization.
405 Additional studies are needed to better understand the occurrence of TeA in grains in
406 relation to the ecophysiology of TeA fungal producers (*E. sorghinum* and *Alternaria* spp.)
407 with worldwide distribution, and to create an integrated management of field and
408 laboratory data to minimizing the risk of this mycotoxin contamination.
409
410

411 **5. Conclusion**

412 This study demonstrated that environmental conditions observed during different
413 growing seasons (summer and autumn) have a direct effect on the TeA contamination of
414 sorghum grains. The average of TeA levels detected in the sorghum samples grown
415 during the summer season was 25 % higher than those detected in autumn. Moreover, the
416 ecophysiological response of *E. sorghinum*, such as mycelium growth, *TAS*1 gene
417 expression and enzymatic production indicated a good agreement with the environmental
418 conditions observed in the field. Hot and humid days seems to play a role in *E. sorghinum*

development and consequently, in TeA accumulation in the grains. The results of the combined approach of the environmental factors under field and laboratory conditions has contributed to create an optimum risk assessment for mycotoxin production in sorghum grains and added fundamental knowledge on the environmental conditions inducing TeA biosynthesis.

Acknowledgments

This work was supported by the Research Support Foundation of the State of São Paulo (FAPESP/Project 09570-1), Coordination for the Improvement of Higher Level -or Education- Personnel (CAPES/Project 006869/2015-07) and U.S. Department of Energy Biological and Environmental Research Science Focus Area grant (grant no. DE-AC52-06NA25396).

Appendix A: Supplementary data

Additional Supporting Information may be found in the online version of this article:

Fig. S.1 Reference gene validation using serial dilutions with reference gene (*TUB2*) and target gene (*TAS1*) and checking for the slope of each line.

Table S.1 Person Correlations of the growth versus gene expression at different temperature and water activity levels

References

1. Codex Alimentarius Commission (2012) CODEX. In: Discuss. Pap. fungi mycotoxins sorghum. ftp://ftp.fao.org/codex/meetings/cccf/cccf6/cf06_14e.pdf. Accessed 6 Mar 2017
2. United States Department of Agriculture USDA Foreign Agricultural Service. In: World Agric. Prod. . https://www.fas.usda.gov/data/world-agricultural-production. Accessed 6 Mar 2017
3. Forbes GA, Bandyopadhyay R, Garcia G (1992) A review of sorghum grain mold. In: Sorghum and millets diseases: a second world review, 1st ed. International Crops Research Institute for the Semi-Arid Tropics, Patancheru, 265–272
4. da Silva JB, Pozzi CR, Mallozzi MAB, et al (2000) Mycoflora and Occurrence of Aflatoxin B 1 and Fumonisin B 1 during Storage of Brazilian Sorghum. J Agric

Food Chem 48:4352–4356 . doi: 10.1021/jf990054w
5. Makun HA, Makun Hussaini A, Gbodi Timothy A, et al (2009) Fungi and Some Mycotoxins Found in Mouldy Sorghum in Niger State, Nigeria. World J Agric Sci 5:5–17
6. González HHL, Martínez EJ, Resnik SL (1997) Fungi associated with sorghum grain from Argentina. Mycopathologia 139:35–41 . doi: 10.1023/A:1006803901969
7. Yekeler H, Bitmiş K, Özçelik N, et al (2001) Analysis of toxic effects of *Alternaria* toxins on esophagus of mice by light and electron microscopy. Toxicol Pathol 29:492–497 . doi: 10.1080/01926230152499980
8. Giambrone JJ, Davis ND, Diener UL (1978) Effect of tenuazonic acid on young chickens. Poult Sci 57:1554–1558
9. Griffin GF, Chu FS (1983) Toxicity of the *Alternaria* metabolites alternariol, alternariol methyl ether, altenuene, and tenuazonic acid in the chicken embryo assay. Appl Environ Microbiol 46:1420–1422
10. Steyn PS, Rabie CJ (1976) Characterization of magnesium and calcium tenuazonate from *Phoma sorghina*. Phytochemistry 15:1977–1979 . doi: 10.1016/S0031-9422(00)88860-3
11. European Food Safety Authority (2011) Scientific Opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. In: EFSA J. http://doi.wiley.com/10.2903/j.efsa.2011.2407. Accessed 6 Mar 2017
12. Asam S, Rychlik M (2013) Potential health hazards due to the occurrence of the mycotoxin tenuazonic acid in infant food. Eur Food Res Technol 236:491–497 . doi: 10.1007/s00217-012-1901-x
13. Rychlik M, Lepper H, Weidner C, Asam S (2016) Risk evaluation of the *Alternaria* mycotoxin tenuazonic acid in foods for adults and infants and subsequent risk management. Food Control 68:181–185
14. Oliveira RC, Davenport KW, Hovde B, et al (2017) Draft Genome Sequence of Sorghum Grain Mold Fungus *Epicoccum sorghinum,* a Producer of Tenuazonic Acid. Genome Announc 5:e01495-16 . doi: 10.1128/GENOMEA.01495-16
15. Delp RB, Stwell LJ, Marois JJ (1986) Evaluation of field sampling techniques for estimation of disease incidence. Phytopathology 76:1299–1305
16. Boerema GH, Gruyter J, Noordeloos ME, Hamers MEC (2004) *Phoma* identification manual. Differentiation of specific and infra-specific taxa in culture. CABI, Wallingford
17. Oliveira RC, Goncalves SS, Oliveira MS, et al (2017) Natural occurrence of tenuazonic acid and *Phoma sorghina* in Brazilian sorghum grains at different maturity stages. Food Chem 230:491–496 . doi: 10.1016/j.foodchem.2017.03.079
18. Gallo A, Solfrizzo M, Epifani F, et al (2016) Effect of temperature and water activity on gene expression and aflatoxin biosynthesis in *Aspergillus flavu*s on almond medium. Int J Food Microbiol 217:162–169 . doi: 10.1016/j.ijfoodmicro.2015.10.026
19. Vaquera S, Patriarca A, Fernandez V (2014) Water activity and temperature effects on growth of *Alternaria arborescens* on tomato medium. Int J Food Microbiol 185:136–139 . doi: 10.1016/j.ijfoodmicro.2014.06.007
20. Raymaekers M, Smets R, Maes B, Cartuyvels R (2009) Checklist for optimization and validation of real-time PCR assays. J Clin Lab Anal 23:145–151 . doi: 10.1002/jcla.20307
21. Oliveira RC, Oliveira RR, de Marchi Bordon C, et al (2013) Enzymatic

characterization of *Corynespora cassiicola* isolates using the API-ZYM ® system. J Phytopathol 161:210–212 . doi: 10.1111/jph.12043
22. Zorzete P, Oliveira RC, Corrêa B (2016) Effects of Conventional and High-Oleic Peanut Medium on Extracellular Enzyme Production and Mycelial Growth of *Aspergillus flavus*. J Food Saf 36:467–473 . doi: 10.1111/jfs.12262
23. Magan N, Medina A (2016) Integrating gene expression, ecology and mycotoxin production by *Fusarium* and *Aspergillus* species in relation to interacting environmental factors. World Mycotoxin J 9:673–684 . doi: 10.3920/WMJ2016.2076
24. EMBRAPA.Empresa Brasileira de Pesquisa Agropecuária (2015) Expansão Potencial da Produção de Sorgo Granífero no Brasil no Sistema de Rotação com Soja Considerando o Zoneamento de Risco Climático 2015/16. https://ainfo.cnptia.embrapa.br/digital/bitstream/item/140133/1/bol-125.pdf. Accessed 6 Mar 2017
25. Srivastava A, Naresh Kumar S, Aggarwal PK (2010) Assessment on vulnerability of sorghum to climate change in India. Agric Ecosyst Environ 138:160–169 . doi: 10.1016/j.agee.2010.04.012
26. Schémaeza B, Somda I, Sereme P, et al (2013) Effects of temperature and pH on mycelium growth of *Phoma sorghina* (Sacc.) Boerema Dorenbosch and Van Kesteren in vitro. Pakistan J Biol Sci PJBS 16:2054–7
27. Lee HB, Patriarca A, Magan N (2015) *Alternaria* in Food: Ecophysiology, Mycotoxin Production and Toxicology. Mycobiology 43:93–106 . doi: 10.5941/MYCO.2015.43.2.93
28. Magan N, Baxter ES (1994) Environmental factors and tenuazonic acid production by *Alternaria* spp. isolated from sorghum. Proceeding 6 Int Work Conf Stored-product 2:1043–1046
29. Oviedo MS, Ramirez ML, Barros GG, Chulze SN (2009) Effect of environmental factors on tenuazonic acid production by *Alternaria alternata* on soybean-based media. J Appl Microbiol 107:1186–1192 . doi: 10.1111/j.1365-2672.2009.04301.x
30. Kubicek CP, Starr TL, Glass NL (2014) Plant Cell Wall–Degrading Enzymes and Their Secretion in Plant-Pathogenic Fungi. Annu Rev Phytopathol 52:427–451 . doi: 10.1146/annurev-phyto-102313-045831
31. Lindner M, Maroschek M, Netherer S, et al (2010) Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. For Ecol Manage 259:698–709 . doi: 10.1016/j.foreco.2009.09.023
32. Pascholati SF (1995) Fitotoxinas e hormônios. In: Bergamin Filho A, Kimati H, AMORIN L (eds) Manual de Fitopatologia, 3rd ed. Agronomica Ceres, São Paulo, pp 365–392
33. Choi J, Kim K-T, Jeon J, Lee Y-H (2013) Fungal plant cell wall-degrading enzyme database: a platform for comparative and evolutionary genomics in fungi and Oomycetes. BMC Genomics 14:S7 . doi: 10.1186/1471-2164-14-S5-S7
34. Yun C-S, Motoyama T, Osada H, et al (2015) Biosynthesis of the mycotoxin tenuazonic acid by a fungal NRPS–PKS hybrid enzyme. Nat Commun 6:1–9 . doi: 10.1038/ncomms9758